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IMMUNE FUNCTION OF IMMATURE RAINBOW TROUT (*Oncorhynchus mykiss*) RELATED WITH PHOTOPERIOD

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Abstract: Radioimmunoassay, enzyme-linked immunospot assay and enzyme immunoassay were used for the determinations of plasma steroid hormone's level, antibody-producing cell's counting and IgM level in this study. The decreased number of antibody producing cells and low IgM levels were observed in sexual immature rainbow trout during the spawning season. These fish were reared under almost constant water temperature and natural photoperiod. Moreover, low IgM level was also observed in immature rainbow trout, which were reared under short photoperiod, and IgM level was not changed by treatment of testosterone. The results suggest that photoperoid may cause the changes in immune competence. It is possible that circadian rhythm accompanied with photoperiod may influence physiological function of fish, so that immune competence is changed.

Key words: Rainbow trout; Immune function; Photoperoid

Seasonal changes, mainly variations of temperature and photoperiod, are known to undergo circadian and circumannual rhythms, and may be the causative agents of variations of the immune functions in lower vertebrates. In goldfish, *Carassius auratus*, reared under natural water temperature and photoperiod, plasma IgM levels had clear annual changes (Suzuki *et al.*, 1996). On the other hand, leucopenia and the decrease in the number of antibody-producing cells and in the level of plasma IgM were observed during the spawning season in mature rainbow trout, *Oncorhynchus mykiss* (Suzuki *et al.*, 1997; Hou *et al.*, 1998, 1999a, b). This suggests that endogenous endocrine rhythms including the synthesis and secretion of steroid hormones may regulate at least in part the activities of immune system of the fish. The bactericidal activity of normal serum decreased during the spawning period of rainbow trout (Iida *et al.*, 1989). Corticosteroids can also regulate the immune activities (Schreck, 1996). It has been demonstrated that administrations of testosterone or other androgens reduce the antibody producing activity of lymphocytes (Slater *et al.*, 1993, 1995; Hou *et al.*, 1999a), and leucocytes *in vitro* may be killed by testosterone (Slater *et al.*, 1997). Wang *et al.* (1994) reported the increased susceptibility of goldfish to *Trypanosoma danileskyi* infection when adminstered with estradiol. In addition, we also know that temperature is one of main factors to influence the fish immunity (Bly *et al.*, 1992). However, it is unclear if photoperoid can cause the variations of immune activities of fish under the conditions that water temperature and steroid hormones remain unchanged.

In this study, we therefore investigated the changes in the number of antibodyproducing cells and IgM level in sexual immature rainbow trout, which were reared under almost constant water temperature and natural photoperiod, and investigated the change in IgM level under different photoperoids.

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1 Materials and Methods

1.1 Experimental project

1.1.1 Effect of natural photoperiod on immune activities Sexual immature female rainbow trout (average body weight 368.7 ± 119.1 g, age 2^+) were used in the study. These fish were reared in a concrete tank with running spring water at 10℃, under natural day light, at the Oizumi Fisheries Research Laboratory of Tokyo University of Fisheries, Oizumi, Yamanashi Prefecture, Japan.

Fish were sampled at about monthly intervals from June 1996 to February 1997. Three to six fish in each time were netted, and anesthetized immediately with 1 000 ppm of 2-phenoxyethanol. Blood was taken from the caudal vasculature with a heparinized syringe within 5 minutes after netting. A portion of blood was allotted for separation of lymphocytes for ELISPOT assay. The remainder was centrifuged at 1 000 × g at 4℃ for 10 minutes and blood plasma was separated. Fish were then weighed and spleen was dissected for separation of lymphocytes for ELISPOT assay. Gonad was weighed and gonado-somatic index (GSI) was calculated. The plasma samples were stored at −25℃ until use.

1.1.2 Effect of different photoperiods on plasma IgM levels Forty juvenile rainbow trout (age 1^-) were obtained from a local farm of Tokyo with body weight from 42 g to 76 g. The fish were reared in 80-liter round plastic tanks supplied with filtered circulating water and photoperiod at 16L : 8D, and fed once daily with commercial trout pellets at a rate of 1% to 2% body weight. After two weeks of acclimation, the fish were equally divided into two groups. One reared under photoperoid of 16L : 8D, and the other under photoperoid of 8L : 16D. Then each group was divided into two subgroups, with one subgroup fed once daily with commercial trout pellets at a rate of 1% to 2% body weight, and the other with testosterone-treated pellets. Plasma samples were initially taken as control. After 30 days, fish were anesthetized and plasma was obtained using the same procedure as above.

1.2 Experimental methods

1.2.1 Steroid hormone radioimmunoassay (RIA) Testosterone (T), estradiol-17 β (E_2), and cortisol (F) were measured by radioimmunoassay (RIA) as described previously (Hou *et al.*, 1998, 1999b).

1.2.2 Isolation of lymphocytes RPMI 1 640 supplemented with L-glutamine, 10% heat inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, was used as the tissue culture media (TCM). Spleen was removed from fish aseptically, and was placed in 5 mL TCM on ice. Single cell suspensions were obtained by teasing the tissue, inhaling and exhaling the tissues through a 5-mL syringe. Peripheral blood and cell suspensions of spleen were applied to a Percoll (Pharmacia) incontinuous density gradient (1.070 − 1.080) centrifugation of 800 × g for 40 minutes at 4℃. Lymphocyte rich fraction at the interface of 1.070 and 1.080 was separated. About 94% of the separated cells were lymphocyte. For the determination of the percentage of the lymphocytes, 0.5 mL cell suspension was used for smear preparation by auto-smear machine. Air-dried smears were stained with Wright and Giemsa stainning. Then smears were examined by microscopy. The identification of the lymphocytes was conducted by Rowley (1990) method. Viable cells were counted with 1% trypan blue as exclusion test (viability > 95%). The cell suspensions were adjusted to be 5×10^6 cells/mL for tissues.

1.2.3 Enzyme-linked immunospot (ELISPOT) assay ELISPOT assay was used for quantifying antibody-producing cells. The ELISPOT assay as the described previously (Davidson *et al.*, 1992) was adapted in this study. Nitrocellulose-bottomed 96-well microtiter plates (Maxisorb, Nunc) were used for the assay. Anti - rainbow trout IgM rabbit IgG and bioti - nylated anti-rainbow trout IgM rabbit IgG were prepared as reported previously (Suzuki *et al.*, 1997). The plates were coated with anti-rainbow trout IgM rabbit IgG (carbonate buffer, pH 9.6), and incubated overnight at 4℃. After washing three times with phosphate buffered saline (pH 7.2) supplemented with 0.05% Tween 20 (PBS-T), wells were blocked

with 5% foetal calf serum (FCS) in PBS for 2 hours at room temperature. The wells were washed twice with PBS-T and once with PBS. Cells were then added to the wells at 5×10^5 cells per well for spleen and peripheral blood. Cultured cells were allowed to secrete IgM in the wells for 6 hours at 18℃. A control was carried out for each cell type, in which the first antibody was omitted. IgM trapped by coated anti-IgM rabbit IgG at the position of the IgM secreting cells was reacted with biotinylated anti-rainbow trout IgM rabbit IgG overnight at 4℃. After washing three times with PBS-T, avidin-biotin-peroxidase complex (ABC Kit, Vector) was reacted for 2 hours. After washing three times with PBS-T, the substrate containing 3, 3′-diaminobenzidine (DAB, Sigma) and 0.04% H_2O_2 (30%) in 0.05 M acetate buffer (pH 5.0) at 100 μL/well were added and allowed for 15－30 minutes at room temperature for development. Then the plates were washed in tap water and air dried. Finally, the number of spots was counted under a microscope and the result was expressed as the number of spot-forming cells per 10^3 lymphocytes.

1.2.4 IgM Enzyme immunoassay (EIA) Plasma IgM was measured by enzyme immunoassay (EIA) as described previously (Hou *et al.*, 1999b).

1.2.5 Statistics The differences of means between the groups were analyzed by Duncan's multiple range test and the differences on means were considered statistically significant at $P<0.05$.

2 Results

2.1 Effect of natural photoperiod on immune activities

The GSI was low and statistical difference was not observed, although GSI increased from December 4 in 1996 and reached the highest (0.24 ± 0.01) on January 24 in 1997. No spawning occurred throughout the experiment. Although plasma T and E_2 levels rose respectively to their peaks in August (7.4 ± 2.5 ng/mL and 5.5 ± 0.3 ng/mL) (Fig. 1), their levels were low, indicating these fish were sexual immature. Plasma cortisol level started to increase from August, and peaked in September (16.7 ± 4.4 ng/mL), then decreased from October onwards (Fig. 1). Seasonal changes in the number of APC in two tissues in female were observed (Fig. 2). The number of APC in peripheral blood decreased from August, then increased from December 25. Plasma IgM levels in females varied in a similar pattern (Fig. 3). The IgM levels started to rise in July, peaked in August (2.83 ± 0.05 mg/mL), then recovered from September.

2.2 Effect of different photoperiods on plasma IgM levels

IgM levels under the photoperoid of 8L : 16D were significantly low than those under 16L : 8D (Fig. 4). IgM levels were respectively 1.82 ± 0.12 mg/mL and 1.76 ± 0.33 mg/mL in control and T-treated groups under 16L:8D, while IgM levels were respectively 1.23 ± 0.29 mg/mL and 1.18 ± 0.26 mg/mL in control and T-treated groups under 8L : 16D. IgM level was 1.77 ± 0.30 mg/mL in initial group. The treatment of T significantly increased plasma T levels comparing with control. Plasma T levels were respectively 3.3 ng/mL and 2.9 ng/mL in the control groups of 16L : 8D and 8L : 16D, while Plasma T levels were 51.8 ng/mL and 58.2 ng/mL respectively in the T-treated groups of 16L : 8D and 8L:16D.

3 Discussion

Some researchers reported seasonal changes in the immune systems of fish, but they attributed the changes to water temperature or sexual maturation (Wishkovsky *et al.*, 1982; Honma *et al.*, 1984). As it is known, water temperature was a factor in controlling plasma IgM levels (Bly *et al.*, 1992; Suzuki *et al.*, 1996, 1997). Cortisol (F) and T were also known to cause lymphocytopenia (Pickering *et al.*, 1987), suppression of antibody-producing cell and IgM (Slater *et al.*, 1993, 1995; Hou *et al.*, 1998, 1999a, b) in salmonid fish. However, the rainbow trout were reared under almost constant water temperature and natural photoperiod, the effects of temperature on fish immune system may be ignored. On the other hand, significant changes in sex steroid hormone levels were observed in sexual immature female

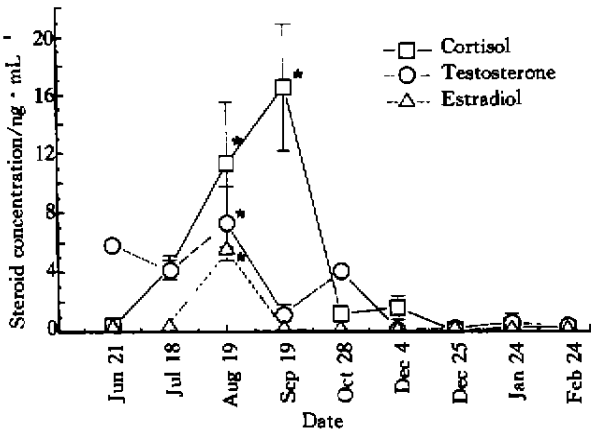

Fig.1 Changes in steroid concentrations of immature female
Each point represents the mean ± SE. * $P<0.05$ compared to the levels of other sampling points on the same line.

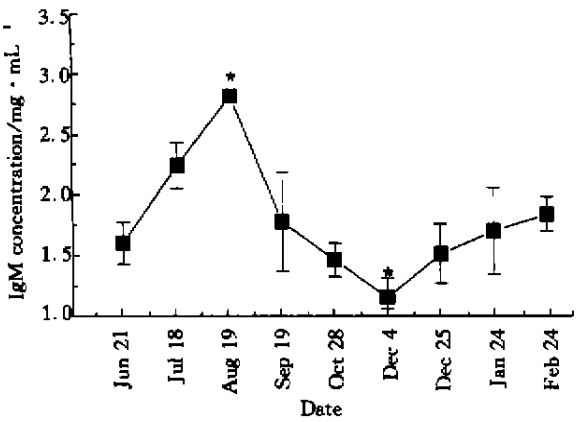

Fig.3 Changes in plasma IgM levels of immature female
Each point represents the mean ± SE. * $P<0.05$ compared to the levels of other sampling points on the same line.

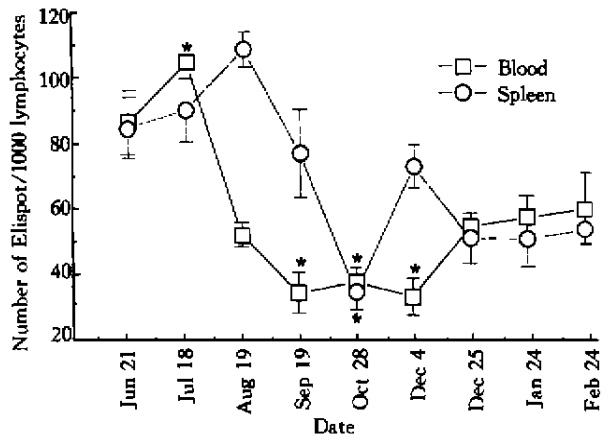

Fig.2 Changes in the number of antibody producing cells (APC) of immature female
Each point represents the mean ± SE. * $P<0.05$ compared to the levels of other sampling points on the same line.

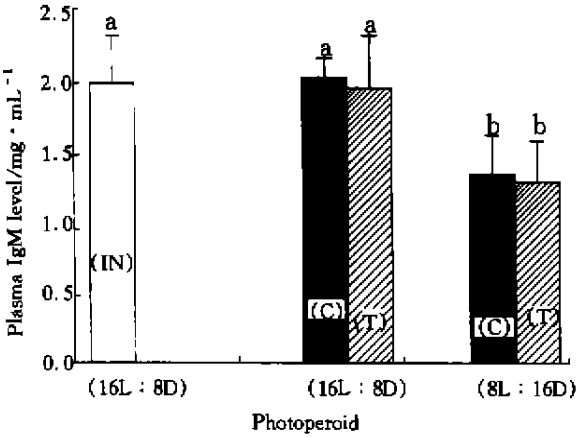

Fig.4 Plasma IgM levels after the acclimation to different photoperoids of 16L:8D and 8L:16D for 30 days
Each point represents the mean ± SE. IN, initial group; C, control group; T, T-treated group. Points sharing the same letter are not significantly different ($P>0.05$).

rainbow trout, but the changes were very smaller than those in mature female. In the mature female rainbow trout, the levels of T and E_2 were above 100 ng/mL and 50 ng/mL (Lou *et al.*, 1984; 1986), while the levels of T and E_2 were below 7.5 ng/mL in sexual immature rainbow trout in the study. This indicates that sex steroid hormones were not related to the changes of immune activities in the study. In addition, F peaked on September 19 and reached about 17 ng/mL, but the elevation was too low to cause the suppression of immunocompetence. These results seem to exclude the effects of the measured steroid hormones on fish immune system, at least in the experiment.

The effects of photoperoid on immunity should be considered in the sexual immature rainbow trout under the condition of constant water temperature. As we know, natural photoperoid becomes short from August on, the decreased immunocompetence just was observed during this season in the study. Short photoperoid may be thought to relate to the low activities of lymphocyte. In order to determine above hypothesis, the effect of different photoperiods on plasma IgM levels was conducted. The results found that plasma IgM levels were closely related to photoperiod when the immature fish were reared under constant water temperature, that is to say, plasam IgM level was low at short photoperiod, while the level was high at long photoperiod. Meanwhile, IgM level was not changed by treat-

ment of testosterone at different photoperiods. These showed that photoperiod can influence immune competence of rainbow trout. However, little is known about the mechanism of effect of photoperiod on immunity in fish, so further study is very necessary.

In conclusion, photoperiod can influence immune functions of fish. There is also a possibility that some other hormones and environment factors may be responsible for the immune changes. We thus would pay an attention to the relationship between environment factors and immune competence thereafter.

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光周期影响雌性虹鳟鱼的免疫活动

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摘要：运用放射免疫测定法、酶联免疫吸附法及酶联免疫斑点法调查了性未成熟雌性虹鳟鱼在生殖季节血浆中的皮质醇、雌二醇、睾丸酮、免疫球蛋白 M（IgM）、抗体产生细胞等水平的变化，同时也测定了不同光周期条件下 IgM 水平的变化。实验结果表明：伴随生殖季节，血浆中甾醇类激素、IgM 量和抗体产生细胞数有季节性的变化；另外，生活在不同光周期条件下的虹鳟鱼，血浆中 IgM 的量明显地不同。由于实验是在常温条件下进行的，而且即使在产卵季节，甾醇类激素水平也没有明显地升高。这些结果提示，与季节变化相关的光周期可能是抑制免疫活动的因素之一。

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